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PARTIAL ANALYSIS OF MILK TRIGLYCERIDES WITH LIPASE IODINE^{131} RETENTION IN CALVES [1]

The microorganism *Geotrichum candidum* (GC) produces an extracellular lipase highly specific for oleic acid in both natural and synthetic triglycerides (TG) (1, 2). Oleate was removed from the latter, regardless of position. The enzyme also differentiated between geometric isomers releasing about 95M% oleate (in the free fatty acids) from glyceryl 1-elaidate 2,3-dioleate and from margarine (7). The utility of the pancreatic lipase method for studying TG structure encouraged us to determine whether GC lipase could be used for the same purpose with milk fat. The results are reported herein.

Butteroil was obtained by churning cream, melting the butter, and filtering the clear oily layer. To provide a standard substrate for comparisons in several of our investigations, quantities of 15-20 g were dispensed in screw-top tubes and stored at -20 C. As needed, samples were melted and purified on an alumina column, the process monitored by thin-layer chromatography (TLC). The butteroil (0.5 g per 8 ml) was dispersed with a Waring Blendor, in phosphate buffer (pH 6.7), containing 1% gum arabic to which were added 0.5 ml of 1% $CaCl_2$ and 25 mg of enzyme preparation in two milliliters of plain phosphate buffer. The enzyme was prepared as described by Alford et al. (2). Two samples, one for column chromatography and one for preparative TLC plus a control containing no enzyme, were incubated with shaking at 37 C for 2 hr. Six runs were made. Synthetic TG's, glyceryl 1-palmitate 2,3 dicaproate (PCC), and glyceryl 1-oleate 2,3 dibutyrate (OBB) were prepared and purified (6). These TG's were mixed (567 mg OBB and 601 mg PCC) and two trials in duplicate run as above. The digestion mixtures from the butteroil and synthetic TG's, respectively, were extracted and the products of lipolysis separated and analyzed as heretofore described, with 4:0 in the FFA determined by column chromatography (3). Net microequivalents of free fatty acid were determined by titration as part of the analysis for 4:0.

The fatty acid composition of the intact butteroil and of the products of lipolysis by GC lipase are listed in Table 1. The specificity for oleate is immediately apparent, as 61.8 M% was present in the FFA as compared to 19.9 in the intact TG's. The low oleate contents of the DG's and MG's reflect this specificity. Very little MG was produced, judging from the relative size of the glyceride bands on the TLC plates. The composition of these MG's does not differ markedly from the MG's resulting from lipolysis of this butteroil by pancreatic lipase (6). However, this comparison is not strictly valid, due to the large difference in activity between GC and pancreatic lipases. For example, 25 mg of GC lipase released 173 microequivalents of FFA from butteroil in 2 hr, whereas 25 mg of pancreatic lipase liberated 300 in 5 min.

From studies of milkfat structure done with pancreatic lipase, most of the 4:0 has been assigned to the 1- or 3- positions, whereas the 2-position has been largely occupied by the saturates (4, 6, 8). If this information is coupled with the data in Table 1, at least one tentative conclusion regarding TG structure can be made. The 4:0 content of the DG's and the 18:1 content of the FFA suggest that TG's of the type; 4:0, S, 18:1 (where S could be 14:0 or 16:0,) exist in milk fat. This tends to confirm the findings of others regarding the presence of this or similar TGs (4, 5).

The presence of butyrate in the FFA prompted us to investigate the action of GC lipase on synthetic glycerides containing 4:0 and 6:0. Results from the lipolysis of a mixture of OBB and PCC are given in Table 2. Data for the MGs are omitted, because these were barely detectable by preparative TLC. Although some saturates were released, most of the FFA was 18:1. More 16:0 than 6:0 was lipolyzed from

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TABLE 1

Fatty Acid Composition (M%) of Products of Lipolysis of Butteroil by GC Lipase

Fatty Acid	Intact TG[a]	Residual TG	DG	MG	FFA
4:0	9.8	11.8	16.6	2.7	5.3[b]
6:0	4.9	6.8	8.3	3.7	0.9
8:0	2.7	2.6	3.7	3.0	1.7
10:0	4.8	4.6	4.6	5.6	1.5
10:1	0.4	0.5	0.4	0.4	0.4
12:0	4.3	4.4	3.9	6.5	1.3
12:1, 13:0, 13:1	0.5	0.3	0.3	0.5	0.2
14:0	11.7	12.7	11.4	19.8	2.7
14:1, 15:0, 15:1	1.6	1.9	1.7	2.2	1.9
16:0	26.8	28.6	26.7	32.5	12.4
16:1	1.9	1.5	1.0	0.8	3.8
17:0, 17:1	0.5	0.2	0.3	0.2	0.9
18:0	9.0	10.4	10.4	13.1	2.0
18:1	19.9	13.1	10.3	8.3	61.8
18:2	0.9	0.5	0.4	0.7	2.9
18:3	0.5	0.1	tr	tr	0.3

[a] Average of six runs.
[b] Determined by column chromatography.

TABLE 2

Lipolysis of a Mixture of OBB and PCC by GC Lipase[a]

Fatty acid	Intact TG		Residual TG	DG	FFA
	Calculated	Found			
	(M %)				
4:0	32.3	31.2	17.1	86.9	6.9[b]
6:0	34.3	33.6	46.7	11.7	2.9
16:0	17.2	16.7	25.8	1.4	8.7
18:1	16.2	18.4	10.5	tr	81.6

[a] Mixture contained 567 mg OBB and 601 mg of PCC. Only a trace of MG was detected.
[b] Determined by column chromatography.

PCC, suggesting a chain-length specificity in addition to specificity for unsaturates. However, this supposition needs to be confirmed with unsaturates of various chain lengths.

At present the application of GC lipase to the study of TG structure of milk fat appears limited. The present preparation is relatively low in activity and requires a long digestion period, which can result in acyl migration. Also, the GC lipase is not absolutely specific for oleate, other unsaturates, as well as saturates, being released (1, 2, 7). While more information is needed on the relative rates of lipolysis of TGs containing oleate in different positions, GC lipase because of the specificity for *cis* 18:1, may possibly be employed in conjunction with pancreatic lipase to analyze fractions of milk fat obtained with $AgNO_3$-TLC or column chromatography.

R. G. Jensen
J. Sampugna
J. G. Quinn
Dorothy L. Carpenter
Department of Animal Industries
University of Connecticut, Storrs
AND
J. A. Alford
USDA, Beltsville, Maryland

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APPARENT IMMUNOLOGICAL SIMILARITY OF BOVINE β-LACTOGLOBULINS A, B, AND C

In the development of a quantitative immunological procedure designed after the procedures of Oudin to determine the β-lactoglobulin content of milk and other materials (7), it was found that rabbit antisera prepared to a mixture of β-lactoglobulin A and B reacted equally well with either the pure A or B form and thus these two proteins appeared to be immunologically indistinguishable. Gough and Jenness (4) and DeWeer et al. (3), using specific antisera prepared against both the A and the B forms in various procedures including the double diffusion Ouchterlony plate tests and immunoelectrophoresis, also indicated the similar immunological identity of the two proteins.

Subsequently, Bell (1) discovered a third genetic polymorph of this protein now called β-lactoglobulin C. Bell and McKenzie (2) concluded from various qualitative immunological analyses that the C form also appeared to be immunologically the same as the A and B forms. However, it has been shown that rabbit antisera prepared to bovine β-lactoglobulins A and B also reacts with the β-lactoglobulins isolated from goat and sheep milk (5) and that goat β-lactoglobulin could not be distinguished from bovine β-lactoglobulin by precipitin tests or by the Ouchterlony method (8). Neverthless, a difference was found between the β-lactoglobulins of these species in the Oudin quantitative diffusion method using antisera to bovine β-lactoglobulin (5). In this procedure a plot of the diffusion distance over the square root of time versus the logarithm of the β-lactoglobulin concentration gave different straight lines for the cow, goat, and sheep β-lactoglobulins. This suggested there existed either some immunological dissimilarity in the determinant groups in each protein or a significant difference in the ratio of the number of immunologically determinant groups per unit weight of the protein. Because of this species similarity, it was found that rabbit antisera prepared to bovine β-lactoglobulin A and B could be used equally well to determine the β-lactoglobulin content of sheep or goat milk with the Oudin procedure by constructing a standard curve using the β-lactoglobulin isolated from each species (5). With the isolation of bovine β-lactoglobulin C, the question arose as to whether it reacted like bovine β-lactoglobulin A and B or exhibited a different quantitative relationship to the antisera. Thus, the following experiments were conducted to evaluate with the Oudin procedure the quantitative relationship of bovine β-lactoglobulin C to the A and B forms.

EXPERIMENTAL PROCEDURE

Oudin analyses were conducted as previously described using capillary tubes, rabbit antisera to β-lactoglobulin A and B, and the β-lactoglobulin samples dissolved at the indicated levels in the pH 7.0 diluting buffer (7). The tubes were sealed, placed at 37 C, and the diffusion zone measured at 24 hr intervals for 72 hr. The diffusion distances divided by the square root of time were plotted versus the logarithm of the β-lactoglobulin concentration and a straight line used to connect through the points.

Ouchterlony analyses were conducted using agar plates (2% Difco Bacto agar in 0.15 M NaCl plus 0.10% phenol) and cutting equidistant wells around a center well filled with the antiserum. The preparatons of β-lactoglobulin were dissolved in the pH 7.0 diluting buffer (7) and placed at various dilutions in the side wells. Readings were made at from three to eight days.

RESULTS AND DISCUSSION

The results shown indicate that there was no perceptible difference found between the three genetic polymorphs of β-lactoglobulin in the Oudin procedure. The variations in the amount found were all within the experimental error of the method (7) and suggest that the three apparently are immunologically identical (Table 1).

It is possible that one of the factors causing slight variation is the small differences in actual molecular weight between the three polymorphs. Amino acid determinations have indicated that β-lactoglobulin C contains two less aspartic acid